Isolation and Cultivation of Mesenchymal Stem Cells from Iliac Crest Bone Marrow for Further Cartilage Defect Management

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ABSTRACT

Aim: *to validate isolation and cultivation methods of bone marrow mesenchymal stem cells (BM-MSCs) from iliac crest, and to compare biological characteristics of BM-MSCs from different age groups for preparation of autologous stem cell therapy in cartilage defect.*

Methods: *patients undergoing spinal surgery were selected and grouped according to age. Iliac crest bone marrow from the patients was aspirated. BM-MSCs were isolated from the bone marrow and then cultivated. Their biological characteristics including morphological appearances and surface biomarkers were evaluated. Growth curves were observed. Sterility and Mycoplasma tests were also performed for quality assessment of BM-MSCs culture procedure.*

Results: *in average, cultivated-BM-MSCs reached the number of 7.56-22.95 x 106 in 4-7 weeks period. BM-MSCs of all age groups showed the same quality of morphology, shape and surface biomarkers (CD105+, CD73+, CD34-, CD45-, CD14-, CD19-, HLA-DR-).*

Conclucion: *our procedures in isolating and cultivating of BM-MSCs have reached required amount for implantation into the cartilage lesion. In addition, the cultivated-BM-MSCs' biological characteristics were also in accordance with International Society of Cell Therapy (ISCT) MSCs criteria.*

Key words: *mesenchymal stem cells, bone marrow, isolation, cultivation.*

INTRODUCTION

Cartilage injury is a common clinical problem that mainly occurs due to cartilage matrix disruption, partial thickness defect, or full thickness defect as a result of traumatic accident.[1] If left untreated, progressive cartilage degeneration could impede mobility and lead to osteoarthritis (OA).[2,3] On the other hand, it is well known that cartilage lesions are irreversible because the capacity of articular cartilage for self-healing is limited.[2-6] Lack of blood supply in cartilage and low metabolic activity of chondrocytes impair natural healing that supposed to fill the defect by increasing hyaline cartilage synthesize activity or stem cell mobilization from bone marrow to site of injury.[7,8] Joint replacement is currently the only treatment option for advanced cartilage damage and for advanced stages of rheumatoid arthritis or OA,[9] yet the procedure should be repeated in 10-15 years ahead. In addition, treatment that offers consistent durable repair cartilage is not yet available.[10] Therefore, transplantation of cells to regenerate the defect in articular cartilage is now an option that could result in longer-term successful regeneration and warrants serious consideration.[9]

First generation of cell transplantation was autologous chondrocyte implantation (ACI) where cartilage defect is covered with in vitro expanded chondrocytes beneath sutured periosteal flap.[11,12] Although hyaline cartilage appeared to fill femoral cartilage defect (1.6 to 6.5 cm^2), this procedure could not regenerate cartilage for a long term, due to loss of flap or cell suspensions.[13] The use of scaffold (e.g.

hyaluronic acid, collagen, biodegradable polymers) was then performed to act as an anchorage for chondrocytes' adherence on cartilage defect, and to promote the secretion of chondrocyte extracellular matrix.[14-16] However, ACI method was quite invasive due to the necessity of surgical procedures for harvesting chondrocytes from non-weight bearing area and implanting chondrocytes at sites of injury.

Recently, the use of human adult stem cells from bone marrow (bone marrow mesenchymal stem cells (BM-MSCs) was widely explored for cell and gene therapy purposes. Human BM-MSCs are relatively easy to be isolated from bone marrow aspirate and expanded in such culture condition, in which they retain their capability to differentiate into several cell lineages including osteoblasts, adipocytes, chondrocytes, myoblasts, and early progenitors of neural cells.[17,18] It has been reported that BM-MSCs also play a role in bone and cartilage homeostasis. Based on these properties, mesenchymal stem cells (MSCs) have been considered as potential cells for cartilage engineering purposes.[18] In addition, the use of BM-MSCs with hyaluronic acid in porcine model has shown cartilage healing outcomes due to appearance of hyaline cartilage after 12 weeks implantation. This study has emphasized the promise of BM-MSCs for managing cartilage injury in less invasive method by using injectable BM-MSCs without surgical procedure.[3]

Although there is only one MSC per 104-105 mononuclear cells in the bone marrow, many researches proved that MSCs can be readily multiplied in vitro.[19-21] According to the International Society for Cell Therapy (ISCT), MSC standard criteria are: 1) adherence to plastic in standard culture conditions; 2) specific surface antigen expression, i.e. positive expression ($\geq$ 95% positive) of CD105, CD73, CD90 expressions, and lack expression ($\leq$2% positive) of CD45, CD34, CD14 or CD11b, CD79α or CD19 and HLA-DR; 3) the ability to differentiate into osteoblasts, adipocytes, chondroblasts under standard in vitro differentiating conditions.[22] Similar to ACI technique, the isolated MSCs have to be proliferated in vitro to increase the cell number. Based on ACI procedures, ~12 millions chondrocytes are required for cartilage defect up to 6 cm,[2,23] while the amount of BM-MSCs for cartilage defect should be more than 7 million.[3] Here we validated our procedure of BM-MSCs isolation and cultivation from iliac crest bone marrow. Our cultivated-BM-MSCs were later tested according to ISCT MSC criteria.[22] To our interest, BM-MSCs' biological characteristics of each age group were compared, to seek whether BM-MSCs of different age group might be difference in the MSCs' biological characteristics.

METHODS

Patient Selection and Bone Marrow Aspiration

Bone marrow aspirates were obtained from patients undergoing spinal surgery in Division of Orthopaedics and Traumatology, Department of Surgery, Dr. Cipto Mangunkusumo Hospital, Jakarta, Indonesia. Inclusion criteria: male/female aged 15-55 years, negative results of HIV, HbsAg, and HCV screening tests. Prior to aspiration, all selected patients were informed regarding this procedure in lay language. Each patient who was willing to participate in this study was asked to sign informed consent. Patients were categorized in 3 age groups: A (15–30 years), B (30–45 years) and C (45–55 years). This study has been approved by Ethical Committee of the Faculty of Medicine University of Indonesia/Cipto Mangunkusumo Hospital, where all bone marrow aspirates were collected. Bone marrow (30 mL) was aspirated from patient's posterior iliac crest and mixed with 5000 U/mL heparin.

BM-MSCs Isolation and Cultivation

One mL of bone marrow aspirate was sampled and sent to quality control (QC) referral laboratory for sterility tests. For BM-MSCs isolation, all procedures were performed in Laboratory of Regenerative and Cellular Therapy (ReGeniC). Bone marrow aspirate was diluted in PBS (1:1) and centrifuged for 30 minutes at 3000 rpm. The resulted buffy coat was isolated, washed, and plated on culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic-antimycotic. The cells were incubated in a humidified incubator at 37^{o}C with 5% CO2 for 5-7 days. The first medium changing was performed on day 5, to remove disturbing cells that might affect the growth of BM-MSCs, and thereafter medium was changed every 2-3 days. Subculture was performed within day 7–10. After 4 weeks, BM-MSCs were harvested. However, extended cultivation can be performed to reach 80-90% confluence. Harvested-BM-MSCs were counted and analyzed further.

BM-MSCs Morphological and Surface Biomarker Detection

The morphological appearances of cultivated-BM-MSCs were observed and documented under inverted microscope (Nikon, Tokyo, Japan) on day 5, 9, 15, 20, and 28. For extended cultivation, documentation was performed when BM-MSCs reached 80-90% confluence. In addition, BM-MSCs surface biomarker detection from each group was performed and compared at the end of culture period. For surface biomarker detection, 1.2x106 BM-MSCs suspension was incubated with PE-conjugated mouse monoclonal anti-CD105 (Abcam, Cambridge, UK), PE-conjugated mouse monoclonal anti-human CD73 (BD Biosciences, San Jose, CA) , FITC-conjugated mouse monoclonal anti-human CD34 (BD Biosciences, San Jose, CA) FITC-conjugated mouse monoclonal anti-CD45 (BD Biosciences, San Jose, CA), FITC-conjugated mouse monoclonal anti-CD14 (Abcam, Cambridge, UK), PE-conjugated mouse monoclonal anti-CD19 (Abcam, Cambridge, UK), or PE-conjugated mouse monoclonal anti-HLA-DR+DP+DQ (Abcam, Cambridge, UK) antibody. To estimate unspecific binding, FITC- or PE-conjugated mouse monoclonal antibody detecting human antigens (BD Biosciences, San Jose, CA) was applied. Detections were conducted with flow cytometry (FACSCalibur, Franklin Lakes, NJ).

Growth Curve Assay

The growth curve assay was conducted by seeding 1,000 BM-MSCs/cm2 in 24-well plate. BM-MSCs were cultured for 24 days, and every 2-3 days growth medium was changed. Cell counting was performed in triplicate.

Bacteria and Mycoplasma Tests of BMSCs Culture

To ensure the safety of BM-MSCs culture, sterility tests for aerobic bacteria and mycoplasma were performed during cultivating period. Supernatant of the cell culture was sampled on day 7 and on harvest day, and sent to independent QC referral laboratory for microbiological testing. For mycoplasma test, PCR-ELISA Mycoplasma Testing Kit (Roche, Mannheim, Germany) was used according to the protocol. Briefly, mycoplasma specific-DNA sequence was amplified with digoxigenin-labeled dUTP (DIG-dUTP) prior to hybridization of single strand PCR products to biotin-probe. Hybridized-amplicon was then immobilized on streptavidin-coated microplate and detected by means of an antibody to digoxigenin coupled with horseradish peroxidase (anti-DIG-POD) and the substrate 3,3',5-5'-tetramethyl-benzidine.

RESULTS

Nine patients were recruited for this study and confirmed negative for HIV, HbsAG, and HCV. Each age group consisted of 3 patients. Within 4 weeks, cultivated BM-MSCs were subjected for harvesting. After 4 weeks of cultivating period, the amount of BM-MSCs isolated from 6 patients was more than 7x106 cells, whereas that from 3 other patients (AKH, SVI and KMA) was less than 7x106 cells, and hence re-seeded until reaching 80-90% confluence (**Table 1**). As the result, in 4-7 weeks all BM-MSCs cultivation were able to generate more than 7 million cells as suggested for implantation in cartilage defect management.[3] Cultivated BM-MSCs showed similar spindle-shape and fibroblastic morphological appearances (**Figure 1**), not only in the observation from day 5 to 28, but also in the observation of extended-cultivation (**Figure 1 and 2**).

Cultivated BM-MSCs in all groups including the extended cultivated cells were confirmed in accordance to ISCT MSCs criteria. All BM-MSCs showed positive surface biomarker expressions of CD105 and CD73, and lack of CD34, CD45, CD14, CD19, and HLA-DR expressions (**Table 2 and Figure 3**). Percentage of CD105 and CD73 expression was ranged between 95.05-99.30% and 97.90-99.10%, respectively, which met ISCT criteria (≥95%). Also in accordance with the ISCT criteria, cultivated BM-MSCs expressed ≤2% expressions of CD34 (0-0.04%), CD45 (0-0.02%), CD14 (0-0.22%), CD19 (0-0.08%), and HLA-DR (0-1.92%).[22]

Table 1. Isolation and cultivation of BM-MSCs from the three age groups (A, B, C)

Group	Subject Initial	Age	# Harvested BM-MSCs on day 28	# Harvested BM-MSCs on extended cultivation period	Culture Period
A	AKH	15	3.40x106	11.90x106	5 weeks
	SVI	15	3.54x106	8.93x106	7 weeks
	STO	21	12.11x106	N/A	4 weeks
B	SVS	30	16.73x106	N/A	4 weeks
	HKY	36	21.70x106	N/A	4 weeks
	SPR	33	22.95x106	N/A	4 weeks
C	ESI	51	7.47x106	N/A	4 weeks
	KMA	48	1.15x106	11.66x106	7 weeks
	HJI	52	20.21x106	N/A	4 weeks

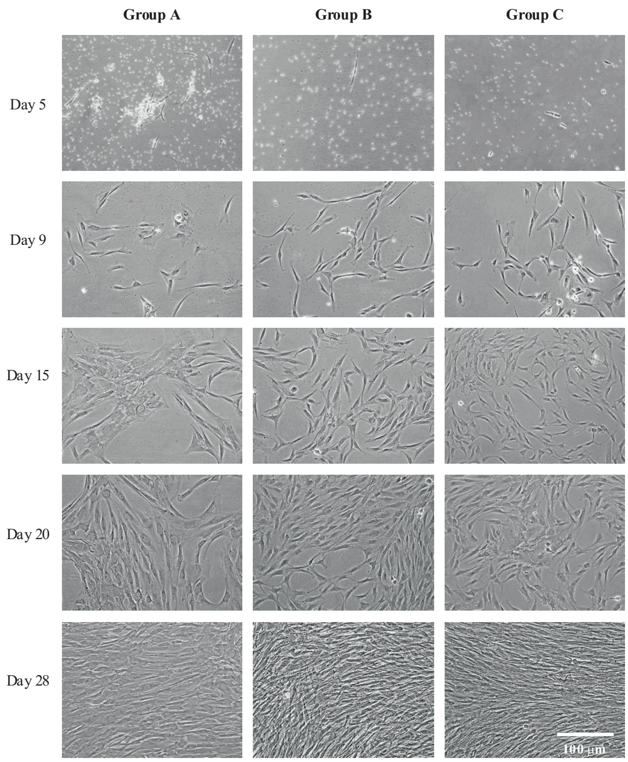

Figure 1. Morphological appearance of cultivated BM-MSCs. The isolated BM-MSCs were cultivated in DMEM containing 10% FBS for 5, 9, 15, 20 and 28 days. BM-MSCs images were captured under inverted microscope (10x10)

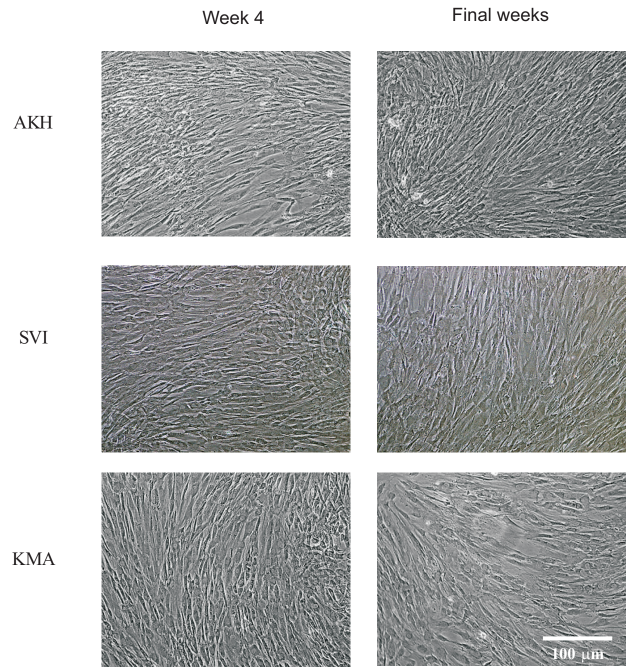

Figure 2. Morphological appearance of BM-MSCs with extended-cultivation. BM-MSCs that did not reach 80% confluence in 4 weeks, were reseeded and re-cultivated in DMEM containing 10% FBS. BM-MSCs images were captured under inverted microscope (10x10)

The growth capacity of all cultivated BM-MSCs groups was equivalent as shown by growth curves in **Figure 4**. All cultivated-BM-MSCs of all three age groups started to enter exponential phase at day 9. As important part to pave BM-MSCs application in cartilage defect management, the absence of aerobic bacteria and mycoplasma of all BM-MSCs culture was confirmed (data not shown).

DISCUSSION

This present study showed that BM-MSCs from iliac crest bone marrow could be isolated and expanded in vitro to reach the required amount of BM-MSCs for cartilage defect therapy. We categorized the samples into three age groups to observe whether age correlated with BM-MSC phenotype in this study since it was reported elsewhere that aging is major limitation in the use of BM-MSCs for repair cartilage lesion due to alteration of BM-MSCs function and capability to home into injury sites.[24,25] Present study showed that

Table 2. Percentage of BM-MSCs surface biomarker expression from the three age groups (A, B, C)

Group	Patient's Initials	FACS Analysis (%)						
		CD105	CD73	CD34	CD45	CD14	CD19	HLA-DR
	AKH	95.36	99.10	0.00	0.00	0.06	0.00	0.00
A	SVI	97.52	97.90	0.02	0.00	0.04	0.00	1.62
	STO	97.08	98.06	0.00	0.00	0.02	0.00	0.00
	SVS	99.3	99.56	0.04	0.00	0.02	0.00	0.00
B	HKY	97.24	98.72	0.00	0.00	0.02	0.00	0.00
	SPR	95.05	98.08	0.02	0.00	0.10	0.00	0.00
	ESI	98.36	99.52	0.00	0.00	-0.08	0.08	1.92
C	KMA	98.08	98.70	0.00	0.00	0.00	0.00	0.00
	HJI	97.3	99.08	0.00	0.02	0.22	0.00	1.2

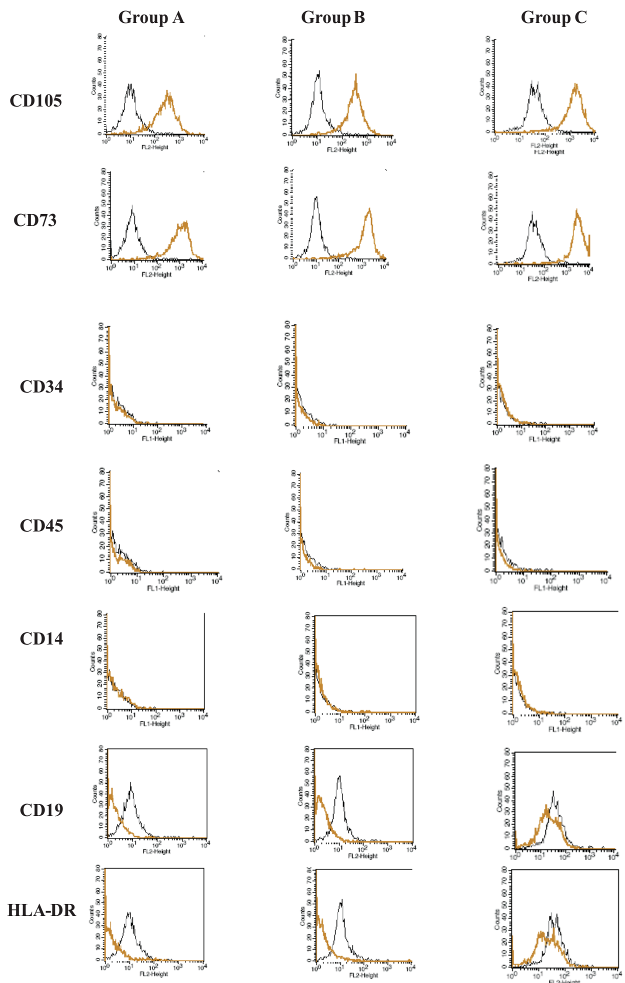

Figure 3. Surface biomarker expression of cultivated BM-MSCs analyzed by FACSCalibur.

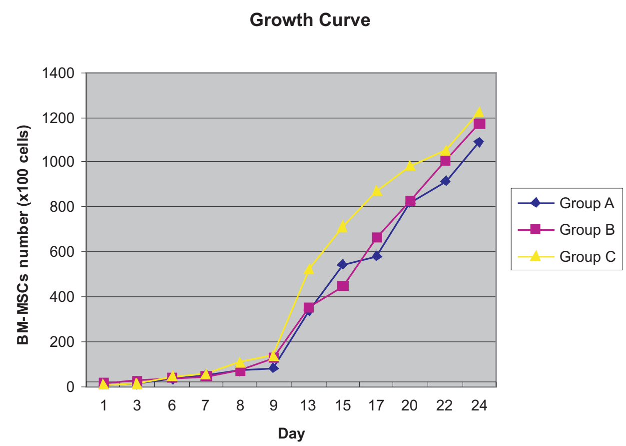

Figure 4. Growth curve of BM-MSCs. BM-MSCs from the 3 age groups were cultured for 24 days and counted in triplicate

BM-MSCs from three age groups, between 15-55 years, showed similar biological characteristics and proliferation capacity. BM-MSCs from all groups met the ISCT criteria for MSC22. They showed fibroblastic morphological appearance with the ability to attach on plastic surface of culture flasks. Their surface biomarker characteristics were shown by positive expressions of CD105 and CD73, while lacking expressions of CD34, CD45, CD14, CD19, and HLA-DR. To fulfill all ISCT criteria for MSC, further study is being pursued to investigate differentiation potential of all cultivated-BM-MSCs.

BM-MSCs from each age group were able to be expanded more than 7x106 cells, which is the required amount for cartilage defect management.[3] As represented in growth curve pattern, achieved amount of cultivated-BM-MSCs occurred due to BM-MSCs proliferation since 9 days adapting period until they reached exponential growth phase. Therefore, our cultivation BM-MSCs method showed its capability to maintain proliferation capacity of isolated BM-MSCs until they were cultivated and ready to be used in further cartilage defect management.

It was observed in this study that for some of BM-MSCs samples (AKH, SVI, KMA), extended culturing period might be necessary to reach the amount of 7x106 cells. The BM-MSCs samples were originated from traumatic spine injury patients and some patients might receive anti-inflammatory drugs. Several in vitro studies reported that anti-inflammatory drugs (non-steroid anti-inflammatory drugs, aspirin) could inhibit mesenchymal BM-MSCs proliferation.[26,27] Therefore, the lower cell amount of these 3 samples did not indicate that BM-MSCs from older patients or Group C could not be expanded as much as the ones from younger patients (Group A & B). As shown on **Figure 4**, the growth curve of oldest group (Group C) was even slightly higher than that of Group A and B. We suggested that variation of BM-MSCs quality among subjects might be effectuated in cultivation of BM-MSCs. Variations in the quality and number of BM-MSCs could happen from different bone marrow aspirates, even when they were obtained from the same donor at the same time.[17] Thus patient's age is not the only factor that may affect BM-MSCs cultivation. Importantly, this study was conducted by cultivating BM-MSCs from patients in the age of 15-55 years old. Hence, BM-MSCs from elder patients were not investigated yet.

Although extension of in vitro cultivation period might result in BM-MSCs senescence,[28] we did not observe any significant alteration in all cultivated-BM-MSCs morphological appearances in this study.

Replicative senescence of BM-MSCs can be observed due to irregular and flat shape.[28,29] Meanwhile, all of our cultivated-BM-MSCs retained their fibroblastic-like cell appearance in small and medium sizes (**Figure 2**). In addition, similar surface biomarker expressions of BM-MSCs, either at 4 weeks or at the extended cultivation period, suggested the persistence of MSCs biological characteristics regardless the duration of cultivation (**Table 2**). Therefore, when it is necessary, cultivating BM-MSCs up to 7 weeks is considered acceptable for the cartilage defect management. Although additional extended cultivation period of BM-MSCs should be avoided as it may decrease the capability of chondrogenic differentiation.[18]

In conjunction with the quality of BM-MSCs preparation, the safety of BM-MSCs for further clinical application is strictly important. The sterility and mycoplasma tests in this study showed that all cultivated BM-MSCs were negative for the tested contaminant agents which pave the safety of BM-MSCs for clinical application.

CONCLUSION

In vitro expansion of BM-MSCs from iliac crest bone marrow of 15-55 years old patients could reach the required amount of 7 millions cells in 4-7 weeks cultivation period. Cultivated-BM-MSCs were confirmed negative for infectious and contaminant agents. Morphological appearances of the BM-MSCs in all subjects were fibroblast-like and in accordance with the first-two ISCT criteria for MSCs. Therefore, we suggest that our protocol for BM-MSCs cultivation could be applied for further cartilage defect management.

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